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Contents

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FURTHER REFINEMENT OF A TECHNIQUE FOR TESTING CONTACT INSECTICIDES[1]

BY W. S. MCLEOD[2]

Abstract

Drosophila melanogaster was sprayed with nicotine sulphate solution by both intermittent and continuous methods. There was no clearly demonstrated superiority of one method over the other. An analysis of variance performed on observed mortalities expressed as anglés of equal information indicated that increasing age of flies, increasing numbers of flies per cage, longer delays between filling of the cages and spraying, and increased proportions of males in the samples raised observed mortalities significantly. The type of cloth used to cover the cages must be standardized. Data on fly ages and numbers of flies per cage were also put through the probit analysis of Bliss, which indicated that flies aged five days were most susceptible and fly numbers affected equally the mortalities due to all concentrations.

Introduction

The historical aspect of the testing of insecticides has already been summarized (17). Among the many contributions to the field since the publication of this summary, two merit especial attention (14, 10).

The present investigation was designed to confirm and enlarge certain of the earlier findings and also to investigate a number of points as yet untouched. It was thus hoped to contribute to a total knowledge of the variable factors in such work and to help make possible the securing of toxicity data reproducible within the limits of the biological variations inherent in the test animal.

Experimental Apparatus and Methods

Breeding Technique

Adult *Drosophila melanogaster* was used as the test animal. The banana shortage necessitated the use of the more laboriously prepared potato medium (16). The amount of yeast was reduced to approximately one Royal yeast cake to 225 gm. of potato. After receipt of Lord's paper (8) emphasizing the importance of the yeast/potato ratio the amounts used were carefully weighed. Yeast cakes were moistened with a minimum of potato water. Food medium was removed from the breeding cages to rearing jars after only

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two days. As soon as the earliest pupation was observed, clean sawdust was added to provide a suitable medium for metamorphosis. Adult flies were held in a clean breeding box, complete with potato-yeast medium, until the population had attained the desired age. It was thought preferable to give the experimental flies full opportunity for oviposition in order to avoid any physiological variations that might be engendered by enforced restraint.

Cages and Covers

All cages were carefully gauged to measure 40 mm. in length by 14 mm. inside diameter. Although this may not have been the best size of cage (10), at least uniformity was maintained.

As 28-mesh tulle was unobtainable, cotton marquisette of 30 strands to the inch was substituted though its fibres softened with washing. Covers were discarded when they became soft. Tests during the course of the work confirmed the importance of this factor.

Method of Filling Cages

The food tray (16) was first removed from a box containing flies of the desired age. A candy jar equipped with a copper-mesh bottom and a special lid designed to fit the window of the breeding box was used to collect the flies. Aside from these slight differences, the equipment and technique were exactly the same as Morrison's (10).

Uniform Sampling

Fifteen flies were placed in each cage and the total mortality from 10 cages was treated as a single variate. Every effort was made to secure a uniform sample in each replicate. Thorough randomization of flies from different cultures was attained by ageing the total spraying population in a single rearing box.

Concluding (11, 8) that sex and physiological condition might influence the speed with which the flies responded to the light (10), it was decided to fill the cages and divide them between the different treatments one at a time in rotation. Since 500 to 1000 flies were discarded daily it was thought that the sex ratio and physiological condition would thus be fairly uniform between variates in any single experiment. This conclusion was borne out by the nonsignificant variance between replicates secured in the experiments on types of cloth covers and on physiological condition when all replicates were taken from the same population.

Spraying Apparatus

The experiments on flies of different ages and on different numbers of flies per cage were conducted with the apparatus and technique used by Morrison (10). One cubic centimetre of liquid was allowed to run into the cup, from which the atomizer sucked it up, and was thus applied to one cage. The cup remained empty but the motor was kept running and hence the pressure in the system was maintained while a new cage was placed in the holder. Another unit of liquid was then added. This has been termed the

intermittent method of spraying as opposed to the continuous method adopted later. By the continuous method, liquid was fed into the cup at a constant rate thus keeping the level there constant while atomization proceeded continuously. The amount atomized onto one cage was kept constant at 1 cc. by regulating the atomizer to deliver this quantity over a definite period of time and by the use of a stop-watch exposing each cage for that required length of time.

Theoretical and mechanical considerations caused the adoption of the continuous spraying technique together with certain minor changes in the method of controlling the rate of spray for subsequent experiments. Once calibrated, the modified apparatus was remarkably constant in performance and the time factor proved an excellent measure of amount of material applied. A stop-watch was used to time all operations and the machine was adjusted to deliver 1 cc. of solution during the 20 sec. exposure per cage.

Duration of Each Experiment

The eight replicates for each experiment were always completed in the shortest possible period of days, it being hoped in this way to minimize any tendencies toward changing susceptibilities in successive generations of the test animal (10, 14). In the experiment on numbers of flies per cage eight replicates were completed in only nine days; on age of test animal, in 13 days; on delays in the daily schedule, 21 days.

Statistical Analysis

Mortalities were recorded 24 hr. after the spraying and transfer of the flies. All flies capable of movement were recorded as living. Percentage mortality was determined to the first decimal for the total dead in each replicate of 10 cages. Mortalities in the check cages were uniformly satisfactory and it was never necessary to correct for this value. Percentage mortalities were transposed to angles of equal information for use in the analysis of variance (4). The data were also plotted graphically as a dosage–mortality curve, using the log. concentration and the probit values of the total mortalities for eight replicates (1, 2, 3).

Experimental Results

Experiment on Effect of Age of Flies

Flies were cleared from the cultures daily between 4 and 6 p.m. On the morning of the following day these were used in the experiment as "flies aged 1 day." Their actual ages, therefore, ranged between 18 and 42 hr. Flies aged three days and five days were handled similarly, their actual ages being 66 to 90 hr. and 114 to 138 hr. respectively. Probably less than 1% of the population was outside these limits.

Each age group was sprayed with a water check and concentrations of 0.5, 0.75, 1.1, 1.25, and 1.5% nicotine. Tables I and II and Fig. 1 give the results of this experiment.

TABLE I

ANALYSIS OF VARIANCE FOR EXPERIMENT ON FLIES *(Drosophila melanogaster)* OF DIFFERENT AGES SPRAYED WITH NICOTINE SULPHATE (EIGHT REPLICATES)

—	Sum of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
For ages of flies	1631.67	2	815.84	23.09	3.09	4.82
For replicates	4407.34	7	629.62	17.82	2.19	2.99
For concentrations	13,681.94	4	3420.49	96.82	2.46	3.51
For error	3745.34	106	35.33			
For total	23,466.29	119				

Standard deviation for error: 5.944°
Interaction of ages and concentrations was not significant

TABLE II

CALCULATION OF THE DOSAGE–MORTALITY CURVES FOR FLIES *(Drosophila melanogaster)* OF DIFFERENT AGES SPRAYED WITH NICOTINE SULPHATE (EIGHT REPLICATES)

—	n'	n	Range of mortality in probits*	$\bar{x}$	$\bar{y}$	b	Chi²
Aged 1 day	5	3	3.49 to 5.03	2.0275	4.5564	3.2273	26.4003
Aged 3 days	5	3	3.61 to 5.09	2.0239	4.6082	3.0940	33.6230
Aged 5 days	5	3	4.06 to 5.27	1.9967	4.8164	2.5552	12.2592

Chi² for 3 degrees of freedom at 5% *P*: 7.815
Chi² for 3 degrees of freedom at 1% *P*: 11.341

** These probit values were read from the calculated regression line at the lowest and highest concentrations used. They are not necessarily the probits of the mortalities obtained experimentally with these concentrations.*

Experiment on Effect of Numbers of Flies per Cage

This experiment was designed to check the evidence of parallelism in Morrison's tests (10) on 15 and 150 flies per container.

Standard cages 40 mm. in length by 14 mm. inside diameter were used throughout the experiment. These were divided into three lots, which contained cages of 16, 32, and 64 flies each, respectively. Since it was impossible to count with perfect accuracy, limits of permissible variation within the lots were set at 12 to 24, 25 to 48, and 49 to 120 flies. A total of 128 flies was considered to be a variate. All flies were aged four days.

Each lot was sprayed with a water check and concentrations of 0.5, 0.75, 1.1, 1.25, 1.5, and 1.75% nicotine. Tables III and IV and Fig. 2 give the results of this experiment.

Usually the regression lines were calculated from probit values based on total mortality from eight replicates of the treatment. This gave one point on the regression line to correspond with each treatment ($n' = 5$ or 6) and

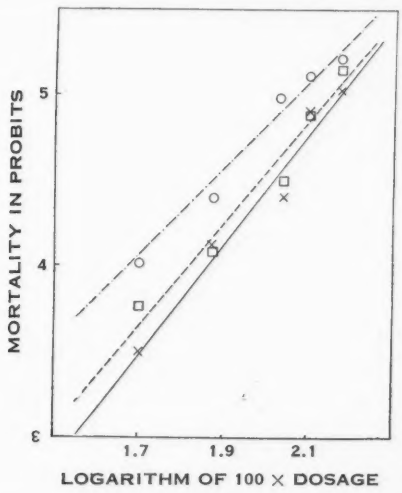

FIG. 1. *Probit-log dosage regression lines for flies (Drosophila melanogaster) of different ages sprayed with nicotine sulphate (eight replicates).* × ———— × *aged 1 day;* □ — — — □ *aged 3 days;* ○ — · — · — · ○ *aged 5 days.*

TABLE III

ANALYSIS OF VARIANCE FOR EXPERIMENT ON NUMBERS OF FLIES *(Drosophila melanogaster)* PER CAGE, USING NICOTINE SULPHATE SPRAY (EIGHT REPLICATES)

——	Sum of squares	Degrees of freedom	Variances	*F*	*F* for 5% *P*	*F* for 1% *P*
For numbers of flies per cage	5814.43	2	2907.22	63.04	3.07	4.78
For replicates	11,108.67	7	1586.95	34.41	2.17	2.95
For concentrations	19,732.68	5	3946.54	85.57	2.29	3.17
For error	5949.41	129	46.12			
For total	42,605.19	143				

Standard deviation for error: 6.791°
Interaction of numbers and concentrations was not significant

the number of degrees of freedom were correspondingly less than the number of points ($n = 3$ or 4, respectively) (3). When each individual test mortality (mortality obtained by one concentration on one day) secured with 4 cages of 32 flies each was treated as a separate point on the probit-log dosage curve the following line parameters were secured:

n'	n	$\bar{x}$	$\bar{y}$	b	Chi²	$\sqrt{2\chi^2} - \sqrt{2n-1}$
48	46	2.0194	5.3440	2.8631	878.0007	32.34417*

(Compare with Table IV)

* *See Table of Chi² in Fisher (7) or Paterson (13).*

TABLE IV

CALCULATION OF THE DOSAGE–MORTALITY CURVES FOR DIFFERENT NUMBERS OF FLIES (*Drosophila melanogaster*) PER CAGE, USING NICOTINE SULPHATE SPRAY (EIGHT REPLICATES)

Flies per cage	n'	n	Range of mortality in probits*	$\bar{x}$	$\bar{y}$	b	Chi²
64	6	4	4.87 to 6.22	1.9874	5.5755	2.6700	26.3605
32	6	4	4.48 to 5.99	2.0197	5.3073	2.5841	39.3267
16	6	4	4.21 to 5.63	2.0295	5.0747	2.6283	25.7383

Chi² for 4 degrees of freedom at 5% P: 9.488
Chi² for 4 degrees of freedom at 1% P: 13.277

* *These probit values were read from the calculated regression line at the lowest and highest concentrations used. They are not necessarily the probits of the mortalities obtained experimentally with these concentrations.*

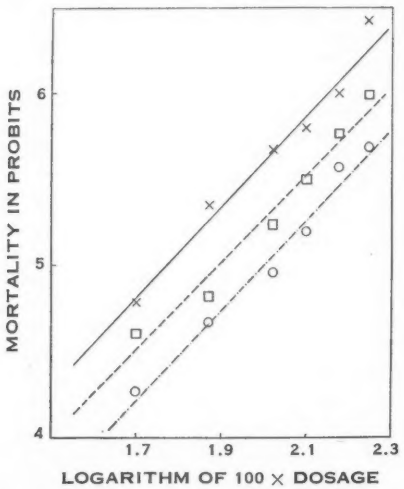

FIG. 2. *Probit–log dosage regression lines for different numbers of flies (Drosophila melanogaster) per cage, using nicotine sulphate spray (eight replicates).* × ———— × *64 flies per cage;* □ — — — □ *32 flies per cage;* ○ — · — · — ○ *16 flies per cage.*

Tests on Rate of Spraying and Amount of Deposit

Cages sprayed with 1 cc. of 1.5% nicotine solution by the intermittent method and then weighed showed a high negative correlation between spraying time and amount of spray deposit. There was no significant correlation between amount of deposit and percentage mortality per cage (Table V).

In view of the high correlation between spraying time and amount of deposit, further weighings were discontinued. Spraying time was measured with a stop watch and correlated against percentage mortality (Table V). Conclusions regarding correlation between amount of deposit and percentage mortality were drawn on the basis of these coefficients.

TABLE V

COEFFICIENTS OF CORRELATION SECURED IN EXPERIMENTS ON RATE OF SPRAYING AND AMOUNT OF DEPOSIT OF SPRAY MATERIALS

Date	Number of cages	Percent nicotine	r	Coefficient of correlation for:
Nov. 18, 19, 20, 21	91	1.5	−0.924	Spraying time × amount of spray deposit
Nov. 20	39	1.5	−0.151	Amount of deposit × percent mortality
Nov. 21	11	0.75	−0.045	Amount of deposit × percent mortality
Nov. 21	29	1.5	−0.075	Amount of deposit × percent mortality
Nov. 30	100	0.9	0.258	Spraying time × percent mortality
Dec. 4	97	0.5	−0.208	Spraying time × percent mortality

Experiments on Intermittent vs. Continuous Spraying

Although data on rate of spraying indicated that minor variations in the intermittent method had no significant effect on mortalities, it was necessary at this time to change to a system of continuous spraying because of the lack of replacement parts for the apparatus. Experiments were therefore undertaken to justify the adoption of the method of continuous spraying.

Daily on three different days 200 cages were prepared, each containing about 15 flies aged four days. Ten cages were sprayed with water and 90 with nicotine by the intermittent method and the remainder were similarly treated by the continuous method. So much work was involved in handling this number of cages that it was necessary to use one spraying technique during the morning and the other directly after lunch. The intermittent method was used during the morning of the first and third days and the continuous method on the morning of the second day. On the first day the cages were recorded in random order and later the individual mortalities were grouped in 10's for statistical treatment. Subsequently the mortalities were recorded in groups of 10 in the order of spraying (Table VI.)

Since morning mortalities were always higher than afternoon, regardless of spraying technique, the effect of delays, during the course of an experiment, on the nicotine solution and the test animal (*Drosophila*) was tested in a short experiment. The results indicated that the nicotine solution did not change in toxicity during a period of nine hours. The F value for delay in the utilization of the flies was high enough to warrant investigation by an adequate experiment. It was interesting to note that mortalities were somewhat higher in the afternoon than in the morning, in contrast with results secured on intermittent vs. continuous spraying.

Experiment on Effect of Delays in the Daily Schedule

For each replicate each of 180 cages was filled with approximately 15 flies, aged four days. These were divided into three lots and were sprayed at 10.30 a.m., 1 p.m., and 3.30 p.m. respectively with a water check and concentrations of 0.7, 0.8, 0.9, 1.0, and 1.1% nicotine at 1 cc. per 20 sec. using the continuous technique.

TABLE VI

STATISTICS FROM DATA SECURED IN COMPARING INTERMITTENT AND CONTINUOUS METHODS OF SPRAYING

Variate	Type of spraying	Total mortality, %	Standard deviation, %	S.d. of the mean, %	Type of spraying	Total mortality, %	Standard deviation, %	S.d. of the mean, %
	Morning spraying				Afternoon spraying			
First day: using 0.8% nicotine, mortalities counted at random and later grouped in order of counting								
Individual cages	Intermittent	37.64	24.11	2.50	Continuous	17.14	18.38	1.92
Groups of 10 cages			5.23	1.74			7.47	2.49
Second day: using 0.8% nicotine, cages sprayed in groups of 10 and groups counted in order of spraying								
Individual cages	Continuous	29.83	32.24	3.22	Intermittent	16.5	15.73	1.57
Groups of 10 cages			6.86	2.17			6.50	2.05
Third day: using 0.9% nicotine, cages sprayed in groups of 10 and groups counted in order of spraying								
Individual cages	Intermittent	54.68	17.94	1.80	Continuous	48.47	17.86	1.79
Groups of 10 cages			3.10	0.98			3.69	1.17

The data were put through the analysis of variance (Table VII) and the provisional regression lines were drawn (Fig. 3) but, since the mortalities were too low to be satisfactory, the calculated regression lines were not obtained nor was the Chi^2 test computed. Table VIII gives the percentage mortality for each treatment based on the total number of flies tested in eight replications. Should the experiment be repeated, higher mortalities would give more reliable data.

TABLE VII

ANALYSIS OF VARIANCE FOR EXPERIMENT ON EFFECT OF DELAYS IN THE SPRAYING SCHEDULE WHEN FLIES *(Drosophila melanogaster)* WERE SPRAYED WITH NICOTINE SULPHATE

——	Sum of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
For times of day	2563.14	2	1281.57	76.30	3.07	4.82
For replications	1747.30	7	249.61	14.86	2.19	2.99
For concentrations	703.21	4	175.80	10.47	2.46	3.51
For error	1780.47	106	16.80			
For total	6794.13	119				

Standard deviation for error: 4.098°

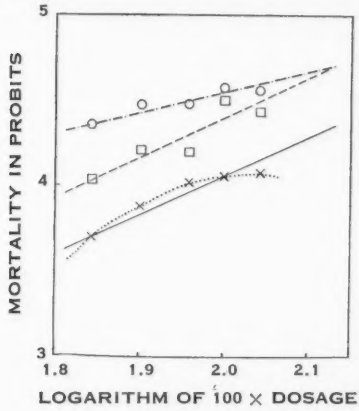

FIG. 3. *Provisional probit–log dosage regression lines for flies (Drosophila melanogaster) sprayed with nicotine sulphate after different periods of delay in the spraying schedule (eight replicates).* × ——— × *flies sprayed at 10.30 a.m.;* □ — — — □ *flies sprayed at 1.00 p.m.;* ○ — · — · — · ○ *flies sprayed at 3.30 p.m.*

TABLE VIII

PERCENTAGE MORTALITY FOR EACH TREATMENT BASED ON TOTAL NUMBER OF FLIES *(Drosophila melanogaster)* SPRAYED WITH NICOTINE SULPHATE IN EIGHT REPLICATES OF EXPERIMENT ON EFFECT OF DELAYS IN THE SPRAYING SCHEDULE

Time of spraying	—	Dosage of nicotine				
		0.7%	0.8%	0.9%	1.0%	1.1%
10.30 a.m.	Total flies	1208	1135	1190	1205	1228
	Mortality, %	9.69	13.39	16.39	17.43	17.43
1.00 p.m.	Total flies	1132	1205	1173	1161	1176
	Mortality, %	16.77	21.33	21.14	30.92	28.57
3.30 p.m.	Total flies	1209	1193	1237	1200	1167
	Mortality, %	26.39	30.18	29.99	33.50	33.08

Experiment on the Effect of Different Types of Cloth Covers

It had been noticed that the marquisette covers on the cages tended to soften with washing. The difficulty had been overcome by constant replacement of worn covers but an experiment on the effect of this factor seemed indicated.

Four types of cloth were used, one on each set of 50 cages; remnants of tulle (10), fine Brussels netting, unused marquisette, and used marquisette. The Brussels netting proved unsatifactory and had to be discarded during the course of the experiment but percentage mortalities on groups of 10 cages from the remaining three types were transposed to angles of equal information and analysed (Table IX).

TABLE IX

ANALYSIS OF VARIANCE FOR EFFECT OF DIFFERENT TYPES OF CLOTH COVERS ON CAGES OF FLIES SPRAYED WITH NICOTINE SULPHATE

——	Sum of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
For different types of cloth	355.45	2	177.73	6.205	4.46	8.65
For replicates	102.78	4	25.70	Not significant		
For error	229.12	8	28.64			
For total	687.35	14				

Standard deviation for error: 5.35°

Experiment on Physiological Condition of the Flies

Physiological condition of the test animal has a pronounced effect on the mortalities secured in toxicity tests, as is shown in the present report by the highly significant *F* tests for replicates in the experiments involving eight replications on different days. It was thought that phototactic responses might be useful in selecting a test population that would give more uniform and predictable results.

Several devices designed to select *Drosophila* from the spraying culture on the basis of their response to light were tried but none proved satisfactory. It was finally decided to clear the culture in the regular manner but without undue disturbance of the flies, which had previously been dark-adapted for 18 hr. Those that rose readily into the collecting jar were classified as "early" flies; those that required to be shaken out were called "late" flies. One hundred cages were filled from each category. Of these, one variate of 10 cages was sprayed with water and the remaining nine variates with 0.9% nicotine. Percentage mortalities were changed to angles of equal information and analysed (Table X).

Test of Homogeneity of Data

Since it had been proved (Table X) that the "early" and "late" flies were of the same population, it was decided to use the percentage mortalities from

TABLE X

ANALYSIS OF VARIANCE FOR EFFECT OF NICOTINE SULPHATE SPRAYED ON TWO LOTS OF FLIES SEPARATED FROM A SINGLE POPULATION ON THE BASIS OF THEIR RESPONSE TO LIGHT

——	Sum of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
For different lots of flies	0.0235	1	0.0235	Not significant		
For replicates	24.44	8	3.06	Not significant		
For error	117.77	8	14.72			
For total	142.24	17				

Standard deviation for error: 3.836°

the 18 variates in these data as a test of homogeneity of results secured by the technique of spraying that had been used. The Chi^2 test of Snedecor and Irwin (15) was used and gave a value of Chi^2 equal to 28.477. (The Chi^2 for 17 degrees of freedom at 5% P is 27.587 and at 2% P is 30.995.)

Experiment on Sex Ratios and the Relative Susceptibility of Male and Female Flies

As soon as mortalities had been recorded in the normal way for the experiment on the physiological condition of the flies, the same cages were counted once again. Both living and dead were sexed and recorded separately in order to give the percentage mortality of male and female *Drosophila*. The work was slow and very hard on the eyes, with the result that it was possible to sex only 1265 flies during the remainder of the day. Since there had not yet been any time to examine the data from the experiment on physiological condition of the flies, it was thought best to select cages from both "early" and "late" flies in case there should prove to be a significant difference between these two categories (Table XI).

TABLE XI

DATA ON RELATIVE SUSCEPTIBILITY OF MALE AND FEMALE *Drosophila melanogaster* WHEN SPRAYED WITH NICOTINE SULPHATE

Group	Total number of flies	Total mortality, %	Number of males	Mortality of males, %	Number of females	Mortality of females, %	Sex ratio males/females
"Early" flies (First flies to leave reservoir)							
1	168	30.7	77	39.0	91	22.0	46/54
2	167	20.0	63	41.3	104	7.7	38/62
3	169	22.9	64	36.0	105	12.4	38/62
4	184	25.9	95	45.3	89	25.3	52/48
Total	688		299		389	Average	43½/56½
"Late" flies (Flies that left reservoir later)							
1	142	22.7	78	30.8	64	12.5	55/45
2	140	27.9	71	39.5	69	17.4	51/49
3	157	35.7	84	52.4	73	15.1	53/47
4	138	28.7	66	33.3	72	15.3	48/52
Total	577		299		278	Average	52/48
Average (eight replic.)		26.8		39.7		16.0	

Discussion of Results

Effect of Age of Flies

The significance of the age factor is unquestionable, since the F value is over seven times as great as the F for 5% P (Table I). An increase in susceptibility was noted as the data on flies aged one, three, and five days were compared, and was especially prominent in the last group (Table II

and Fig. 1); however, the Chi^2 test indicated greater uniformity of results in flies aged five days. It might be worth noting also that Morrison (10) secured a smaller Chi^2 value for four- and five-day than for three-day–old flies. In any case the author agrees with Nelson *et al.* (12) that flies of any age are equally satisfactory, provided that the particular age chosen is used consistently throughout the experiment.

Since Morrison and others who used the banana medium cleared their culture jars early in the morning of the day of the experiment, their one-day–old flies ranged from 0 to 24 hr. while, as previously explained, those flies listed herein as "aged one day" were actually from 18 to 42 hr. old. This will account for the fact that the writer did not secure indications of such high susceptibility in the lowest age group as did some of the other workers.

It will be noted in Fig. 1 that the slope of the regression line for flies aged five days is considerably less than corresponding values for either three-day or one-day flies, being 2.55 as compared with 3.09 and 3.22 respectively. Should these values be confirmed in a repetition of this experiment it would mean that the comparative susceptibility of flies aged five days is increased by a greater amount in the case of the lower concentrations than of the higher ones. This will indicate that, in the absence of parallelism of the regression lines, results of experiments with the same toxicant on different ages of *Drosophila* may not be truly compared at any one level of mortality. It will thus be possible to make such comparisons only by means of the parameters for slope and position of the regression line.

Effect of Numbers of Flies per Cage

Morrison (10) found that mortality steadily increased with increases in the number of flies per cage. He suspected but did not demonstrate a parallelism between the regression lines for different numbers per container, using the spraying technique. In addition, his Chi^2 values indicated less heterogeneity in the data when the smaller numbers were used.

Stultz (16), who used lantern globes as cages, stated, "Spray tests with nicotine sulphate . . . . . do not indicate any significant correlation between number of flies per globe and percentage kill."

The experiment herein recorded definitely supports the findings of Morrison. The F value for numbers of flies per cage (Table III) is over 20 times the value of F for 5% P. This degree of significance cannot be doubted. Furthermore, the parallelism between the regression lines is also demonstrated within close limits (Table IV and Fig. 2) since the values of b, the slope, for 16, 32, and 64 flies per cage are respectively 2.63, 2.58, and 2.67, when determined on the basis of six points. The Chi^2 values (Table IV) are also of interest since that for 16 flies per cage is comparatively satisfactory though it should be noted that the value for 64 flies per cage is almost as low.

Such evidence makes it necessary that Stultz's statement be qualified in some manner. It may be that a significant correlation exists only when some factor such as absolute size of the cage, or average volume of cage per fly,

is below a certain threshold value. What this factor may be the author did not discover but it would seem that one exists and that Stultz was working outside the limits of its effect. The parallelism of slopes in the regression lines allows us to conclude that this undiscovered factor acts with equal intensity regardless of the concentration of chemical being used.

The method of using the mortality from each concentration in each replicate for the calculation of the regression line and the value of Chi^2 is not to be advocated for comprehensive experiments. For one thing, the calculation is considerably more laborious. Moreover, the increase of Chi^2 due to the use of many points about the regression line was not in any way compensated for by the increase in the number of degrees of freedom. Consequently the value of 32.344 secured with 46 degrees of freedom, from analysis of individual test mortalities of cages containing 32 flies each, was 16 times the value of 2, which is considered to be satisfactory, while the Chi^2 value of 39.327 secured from the same data using only 4 degrees of freedom (Table IV) was less than $4\frac{1}{2}$ times the value of 9.488 obtained from the Chi^2 table at 5% *P*. This demonstrated that, when drawing a regression line in toxicity tests, the method of plotting the mortality secured from the totals of many replicates is easier and gives a lower value of Chi^2 than does the method of plotting the individual values secured in each replicate. This is natural enough since the averaging of the results from a number of replicates tends primarily to remove the effects of extreme variation in some of them.

Effect of Variations in Spraying Technique

Callaway and Musgrave (6) kept concentration of the insecticide constant but secured variations in effect by varying the amount of deposit. They found that percentage mortality increased with increased deposit. Potter (14) also found that percentage mortality increased with increased deposit, provided the concentration being applied was above the lower threshold of toxicity.

Morrison (10) found that rate of application was not an important factor within the limits tested, though he adopted the slower rate on the basis of a lower Chi^2 value and lower variance in the data. He did not actually measure the deposit. The writer, in similar experiments, found that 1 cc. of 1.5% nicotine sprayed in 10 sec. gave an average deposit of 70 mg. while the same quantity sprayed in 25 sec. gave an average deposit of 48 mg. It is logical therefore to assume that Morrison actually varied the amount of deposit without securing a significant effect.

The writer found results similar to Morrison's when using the intermittent spraying technique. The problem was not fully investigated nor was it investigated at all after the adoption of the constant spraying method. This latter would, it is thought, prove to be an excellent method for such an investigation.

In this experiment the actual amount of spray material placed in the reservoir was always constant at 1 cc. per cage. The amount reaching the cage and, it is presumed, the test insects was measured at first by weighing. This amount was found to depend directly on the condition of the jet. When

the latter was clean, the liquid was quickly applied in relatively large droplets with a consequently heavy deposit. As the jet became somewhat clogged with nicotine the spray required much longer for atomization, the droplets became much finer, and less of the resulting mist became deposited on the cage and its contents. Although the application was relatively much heavier than that secured by Potter (as much as 80 mg. on a cage 14 mm. in diameter as compared with Potter's deposits of up to 140 mg. on a Petri dish 9 cm. in diameter) and the concentration of 1.5% nicotine was definitely high in the toxic range, the coefficients secured failed to show any significant correlation between amount of deposit and percentage mortality. Further work correlating spraying time (which varied inversely as the weight of deposit, having a coefficient of correlation of -0.924) with percentage mortality resulted in correlation coefficients of greater numerical size but still not large enough to be considered significant (Table V).

It is probable that Potter's statement is true as far as it goes. He says that "Where the concentration of the poison is below a certain threshold value, variation of the deposit within wide limits has little or no effect." He goes on to state that above this threshold the mortality is affected by the weight of deposit. It seems possible, then, that weight of deposit as well as concentration of poison may have different effects in different ranges. The amounts Potter used (ranging up to 2.4 mg. per sq. cm. when calculated on the basis of 155 mg. on a 9 cm. Petri dish) were within the range where variations in deposit have an effect on percentage mortality while the amounts used by the author (ranging up to about 52 mg. per sq. cm. when calculated on the basis of 80 mg. on a cage 1.4 cm. in diameter) were so far above this range that variations in amount of deposit had little or no effect.

In the comparison of intermittent and continuous methods of spraying a review of the data in Table VI leads to a conclusion that there is no clear evidence for the superiority of either method. The standard deviations of the experiments and the standard deviations of the means are comparable in magnitude. In any case, evidence had already been secured that the spraying rate was more constant for each 1 cc. portion when using the continuous method but further evidence indicated that amount of deposit, within the range of applications recorded in this work, had little effect on mortality. The author feels justified in concluding, therefore, that the substitution of continuous for intermittent spraying would certainly not introduce any greater heterogeneity in the results of experiments and would provide some possibility of improvement in the consistency of the data.

The most interesting observation was that mortalities were consistently higher in the morning than in the afternoon, regardless of the type of spraying being used. This was the opposite of what would naturally be expected and further experiments failed to discover any reason or explanation for the phenomenon.

Effect of Delays

In this experiment the author was led to the choice of these concentrations by results secured previously (Table VI, third day) when 200 cages sprayed with 0.9% nicotine produced an average mortality close to 50%. The only explanation for the low mortalities secured in the later experiment on effect of delays is that the breeding cabinet was fumigated with nicofume during the intervening period. Though subsequently well ventilated, the cabinet may have retained enough nicotine to kill the weaker flies with the result that these tests were actually run on what was for all practical purposes a stock of flies selected on the basis of resistance to nicotine.

In any case, the *F* test for delays in the daily schedule ("times of day", Table VII) was highly significant and there can be no doubt that such delays have a marked influence on results secured in laboratory tests with insecticides, particularly when the test animal is unable to get along without food and water for a period of 24 hr. The obvious correction for this factor is to make all haste with the experiment so as to finish it within the shortest possible period of time and to randomize the order of application of treatments on different days of a replicated experiment. All parts of the work should be done on a strict time schedule and deviations from this schedule should be eliminated as far as possible. Should any unforeseen difficulties cause a serious loss of time during the experiment, the results should not be accepted until a careful scrutiny proves that the mortalities are comparable to those secured in other replicates.

The regression lines in Fig. 3 and the percentage mortalities in Table VIII indicate that a progressive increase in susceptibility took place as the period of delay increased in length. This is the result one would naturally expect and the weight of evidence from eight replicates in this experiment should be sufficient together with a similar result secured in the preliminary experiment to overbalance the unexplained results secured in the three tests on intermittent vs. continuous spraying (Table VI). It will be noticed that the points for flies sprayed at 10.30 a.m. follow a definite curve but no conclusions may safely be drawn from any of the provisional regression lines because of the low mortalities secured.

Effect of Different Types of Cloth Covers

Although this was not a comprehensive test, the fact that the *F* value secured was greater than *F* for 5% *P* (Table IX) indicates that the use of different types of cloth in covering the cages may have a significant effect on the mortalities secured. If this is so, it is logical to conclude that a cloth such as marquisette, which softens with continued use, may bring about a progressive change in the level of mortalities secured. In such a case the only safe procedure is to discard the covers after a certain number of tests, before the

softening has proceeded far enough to influence the results. Tulle, if available, is a much more satisfactory material.

Homogeneity of the Data

Throughout this work and also in a review of the literature, it was found that smaller samples tended to produce smaller values of Chi^2. Callaway and Musgrave (6) secured a measure of homogeneity in five tests that consisted of 14, 7, 8, 11, and 8 determinations respectively while heterogeneity was demonstrated in three tests that consisted of 40, 20, and 19 determinations. Each point was determined by a test of less than 100 eggs. Potter (14), using about 50 or 100 insects in the determination of each point on his regression lines, secured values of Chi^2 that were in general very satisfactory, indicating definite homogeneity in all except one of his tests. Morrison (10), on the other hand, using 8 or 10 replicates of about 150 flies each, a total of 1200 to 1500 flies for each point about the regression line, secured indications of heterogeneity in all tests except one. The author, using eight replicates of 150 flies each, found similar indications of heterogeneity.

Morrison stated: "The very large Chi^2 suggests that the theory involved in the application of the method of probits probably does not describe the data and some curve other than a straight line would fit the converted data better." There seems to be opportunity for research on this point. Moore and Bliss (9) plotted the regression lines of seven different chemicals each replicated three times in tests on aphids. On the basis of this work they stated: "Inspection of the diagrams for the individual series indicated no systematic departure from linearity. The plotted points either adhered closely to a straight line or followed a concave as often as a convex trend." Upon combining all of these lines into one composite line, they said, "It is apparent from the figure that there was no consistent nonlinear trend which would warrant shifting to some transformations other than the log-concentration and the probit."

Wadley and Sullivan (18) studied the dosage–mortality curve and concluded that "Linearity was positively disproved in this material. It seems likely that the log-probit transformation is useful but not perfect, ... and that further study of the curve is justified."

Although they maintained the suitability of the straight line as a measure of the probit–log dosage relationship, Moore and Bliss (9) found values of Chi^2 indicative of heterogeneity in 13 of the 21 series and "a highly significant heterogeneity for the experiment as a whole." In explanation of this fact, they stated: "The number of aphids per pot averaged more than 560. So large a number reduced the sampling error in estimating the percentage of dead aphids on the plants of each pot to a relatively small value and exposed the heterogeneity of the four points about their computed curve." This, then

would also seem to be the explanation of the high values of Chi^2 found by Morrison (10) in his tests and by the author in the experiments on age of flies and numbers of flies per cage. Nevertheless it would seem that there is some defect in the statistical method. Either the basic premise of normality of distribution is not sound or the Chi^2 is not a satisfactory measure of homogeneity of the data and should not be used for such a purpose. One would naturally expect the measure of homogeneity to be more satisfactory with larger samples yet such is not the case in the probit–log dosage method.

In contrast to the high values of Chi^2 in the two experiments where so many flies were tested, we may look at the value secured when 18 variates, each consisting of only 150 flies, were put through the Chi^2 test of Snedecor and Irwin (15). In this test the Chi^2 of 28.477 is about at the level of 4% P which is well above the 1% P accepted as being satisfactory by such workers as Murray (11) and Callaway and Musgrave (6).

Again in their discussion of heterogeneity, Moore and Bliss say, "Conclusions depend primarily, therefore, upon differences between curves and the consistency of these differences rather than upon inferences drawn from the degree of their internal homogeneity." It seems a valid conclusion, therefore, that the use of large numbers of flies in establishing a point on the regression line is not unjustified although the Chi^2 values secured will be quite high. At least such a procedure will have the effect of reducing the sampling error and the resulting line will be more satisfactory for work in comparative testing of contact insecticides than if it were based on smaller samples.

Sex Ratios and Relative Susceptibility of Male and Female Flies

Murray (11) working on houseflies and Lord (8) on *Drosophila* both found males predominating in the first cages filled from any single population of flies. The author, on the basis of only one day's work, was unable to confirm this fact but it is probable that the reversal of sex ratios (Table XI) was due to chance. Murray and Lord make no mention of the condition under which the flies were kept previous to the experiment and the ratios recorded in Table XI may have been secured as a direct result of the fact that the flies had been kept in absolute darkness for a period of 18 hr. just before the experiment.

With regard to the relative susceptibilities of the two sexes there is no disagreement. All three experiments demonstrate the greater susceptibility of the male.

It is evident, therefore, that the matter of sex ratio of the test animals is one of considerable importance and probably contributes in a marked degree to variations in the mortalities secured. It will be necessary for this reason to devise some method of reporting the susceptibilities separately according to sex (as suggested by Murray) or of securing constant sex ratios between variates in the experiment.

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